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Rapid Synthesis and In Situ Screening of Potent HIV-1 Protease Dimerization Inhibitors

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Summary

A library of dimerization inhibitors of HIV-1 protease is described based on crosslinked interfacial peptides. The 54 component library was designed to contain two modifications to the starting structure, one each in the Northern and Southern fragments. A rapid synthesis and in situ screening method in microtiter plates was developed to facilitate the generation and evaluation of the library members. More than 90% of the doubly modified agents were more potent than their respective singly mutated parent compounds, and five of the most potent dimerization inhibitors of HIV-1 protease described to date were identified. The free energy of binding for the combined two modifications was generally found to be additive, demonstrating the predictive value of earlier libraries.

Introduction

In the search for novel inhibitors to block HIV infection, HIV protease (PR) is a target of great importance. In addition to the many classes of agents that target the active site [1–3], inhibitors that bind the dimeric interface of PR have been identified [4]. In the latter category, crosslinked interfacial peptides of PR have been shown to inhibit the dimerization and activity of PR [5, 6]. Recently, a library of agents, based on the structure of inhibitor **1**, was studied [7]. The library was composed of single amino acid modifications to **1**, and fairly potent dimerization inhibitors were identified.

To improve potency, simultaneous and multiple modifications to inhibitor **1** would be desirable. A number of the best, single amino acid changes to **1** could be used to enhance diversity with the potential to improve efficacy. Even a small library of this sort, however, is time consuming to synthesize, purify, and screen. The in situ synthesis and screening of sulfotransferase [8], α-fucosidase [9], and active site HIV PR inhibitors [10] has been reported by Wong and coworkers. In these studies, an amide bond was formed as the last step of the inhibitor synthesis in microtiter plates, and the agent was screened without purification. A similar amide bond-forming reaction was envisioned for the preparation of HIV PR dimerization inhibitors. Herein we describe a synthesis and in situ screening method to evaluate a focused library of agents based on inhibitor **1** that contain double modifications to the structure.

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Results and Discussion

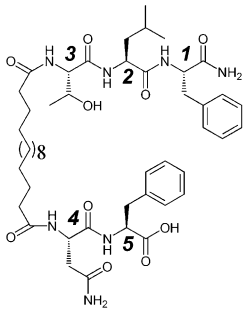
Selection of Library Components

Compound **1** (Figure 1) is composed of a Northern tripeptide fragment and a Southern dipeptide fragment linked at their amino termini by an aliphatic tether. Three modifications were chosen for each of the five positions within **1**, based on the previous library of single amino acid changes (Figure 1). Since aromatic and cyclic aliphatic moieties were found to be essential at positions *1* and *5* of compound **1**, the most effective side chains were included in the library design [7]. Likewise, the branched aliphatic side chains that were found to be most effective within position *2* were accommodated as library components. Positions *3* and *4* both preferred a phenyl side chain; however, to improve the solubility of members of the library, branched aliphatic and more hydrophilic groups were included. Two simultaneous mutations were planned within the structure of **1**, one in the Northern fragment and one in the Southern fragment. The combination of nine Northern components and six Southern components, therefore, would lead to 54 unique library members.

In Situ Synthesis and Screening of Library

Previous syntheses of crosslinked interfacial peptide inhibitors of HIV PR dimerization have been carried out in solution and on solid support [5, 6]. In both cases, extensive purifications were performed prior to testing. It would be of interest to develop methods in which the final compounds could be tested in situ, because compound libraries are time intensive to purify. Recently, Wong and coworkers described libraries of compounds by using an in situ amide bond-forming reaction between the amine and carboxylic acid components with HBTU coupling [8–10]. The free acid form of compounds such as **A** (Figure 2) had poor solubility. Therefore, it was expeditious to generate Northern fragments (**A**) with the NHS-ester intact for reaction with the Southern dipeptide amines (**B**) (Figure 2). In a typical reaction, solutions of the Northern fragments (**A**) in DMSO in microtiter plates were treated with solutions of the Southern dipeptides in DMSO (**B**, 4 equivalents) and DIEA. Reactions were monitored by HPLC and mass spectrometry for complete consumption of **A** (10–12 hr), and, with the exception of residual **B**, NHS, and DIEA, the desired material was formed in greater than 95% purity.

For in situ screening, it was essential to determine if the reaction byproducts and excess reagents had inhibitory activity. All Southern dipeptides were tested against HIV PR up to a concentration of 300 μM, and less than 10% inhibition was observed in all cases. Likewise, NHS and DIEA at concentrations of 300 μM had no effect on HIV PR activity, nor did combinations of these two reagents with the Southern peptides. These data were a good indication that the crude library components would have similar activity to those that were purified. To test this hypothesis, we purified ten of the library members to homogeneity by HPLC and compared their IC_{50} values to those of their in situ-generated



(1)

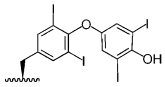
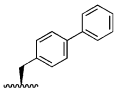
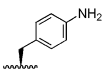
Northern Components			Southern Components	
Position *1*	Position *2*	Position *3*	Position *4*	Position *5*
 Thx1	 Chg2	 Leu3	 $CmeO_2$4	 Hfe5
 Chg1	 Ile2	 Met3	 Met4	 Cha5
 Bip1	 Cha2	 Val3	 Paf4	 Nal5

Figure 1. The Modifications Chosen at the Five Positions of Compound 1 for the Double Mutation Study

counterparts (see the Supplemental Data available with this article online). In the latter case, the reaction mixtures were diluted to 400 μM in the desired compound and transferred to a new microtiter plate. The fluorogenic assay of Toth and Marshall [11] was used to determine inhibitory activity against HIV PR. In all cases, the IC_{50} values obtained were either within experimental error or were within 10% of each other for the purified or in situ-generated compounds. The success of this experiment prompted us, therefore, to evaluate all 54 compounds in situ against HIV PR. Both compound **1** and Thx1 were reevaluated concurrently with the 54 compound library, and the values obtained were found to be within the errors of the previously reported values [6].

Interesting trends can be discerned from analysis of the inhibitory activity of the doubly modified library members (Table 1; Figure 3). First, all of the inhibitors of the library were more potent than the starting compound **1** (IC_{50} of 5.5 μM, K_i of 3.0 μM). Also, more than 90% of the double-modified compounds were either more potent or equipotent as compared to their singly mutated parent compounds. Overall, the most improved compound was Chg1Nal5; this compound was 10-fold and 9-fold more potent than its two parent inhibitors with single mutations (Chg1 and Nal5, respectively). The most improved series of compounds was found within position *5*. Each of the single mutant inhibitors (Cha5, Hfe5, and Nal5) was a moderately potent agent that was improved, on average, 7.5-fold with an additional modification to its structure. The least improved series based on the Northern fragments included those compounds containing Thx in position *1*; for instance, equivalent activity was obtained with Thx1Hfe5, whereas a 1.7-fold increase was observed with Thx1Nal5, as compared to Thx1. Thx1 was already a potent single mutation, and modification to the other four positions had less of an effect overall. Likewise, additional mutations to the Paf4 compound were less productive than that observed for the other Southern peptide changes. The three most potent agents from the double mutation studies, however, were those that contained Thx1 in addition to mutations at Paf4, Cha5, and Nal5.

Seven of the more potent agents from the in situ screening were analyzed further to determine K_i values and their mechanism of inhibition. All compounds were found to be dimerization inhibitors by the kinetic method of Zhang and Poorman [12], as a plot of $E_o/v^{1/2}$ versus $v^{1/2}$ led to parallel lines for HIV PR with and without inhibitor (Figure 4). Additionally, five compounds were found to be among the most potent dimerization inhibitors of HIV PR that have been reported to date, with K_i values between 30 and 50 nM (Table 2).

We were interested in determining the predictive value of our single mutant library for future multiple mutation libraries. Therefore, we compared the K_i values obtained for the doubly modified inhibitors to the K_i values for each of their two parent compounds to determine what effect the two simultaneous changes had on inhibition. We calculated the increase in potency that should be observed, as compared to inhibitor **1**, if the two modifications had an additive effect (Table 3). These data were then compared to the observed increase in potency over compound **1**. In three cases, the value that

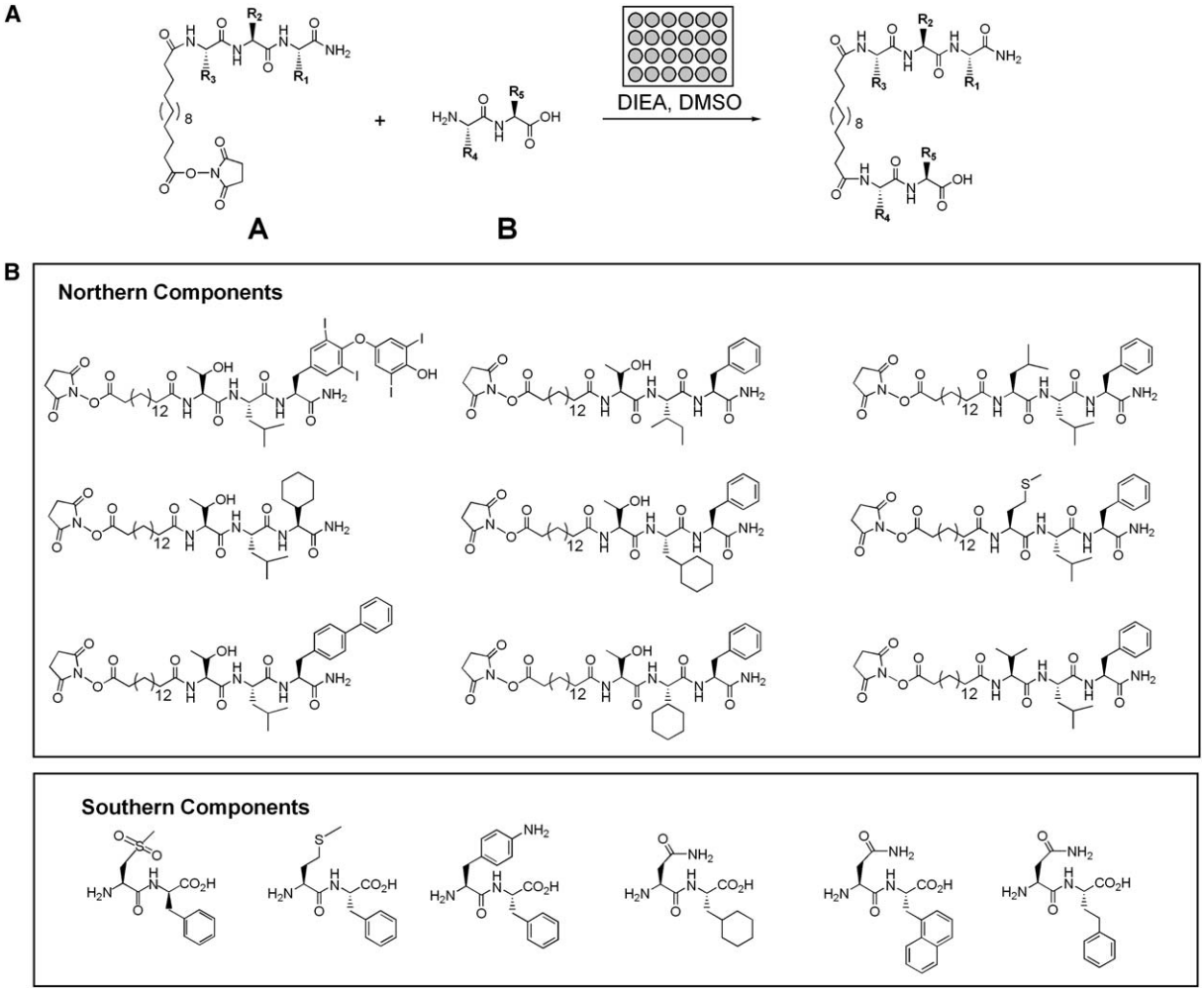


Figure 2. Microtiter Plate Synthesis

(A) Schematic of the reaction of the acylated Northern peptide fragments and Southern peptide fragments in a microtiter plate format.
(B) Structure of the nine acylated Northern fragments and six Southern fragments used to compose the doubly modified library.

was predicted, assuming additivity of the free energies of binding, was within experimental error of the actual increase in potency over compound **1**. In two cases, the actual value was within 10%–20% of the calculated increase in potency, and, in two cases, there was a major discrepancy with the predicted value. Thx1Paf4

Table 1. IC_{50} Values, μM, of the Doubly Modified Library Members against HIV-1 PR

		Southern					
		Position *4*			Position *5*		
Northern		$CmeO_2$ (2.9)	Paf (0.6)	Met (2.7)	Cha (3.7)	Hfe (4.4)	Nal (2.9)
Position *1*	Thx (0.4)	0.46	0.24	0.41	0.24	0.4	0.23
	Bip (1.09)	0.51	1.02	0.46	0.69	0.64	0.51
	Chg (3.1)	0.58	0.90	0.67	0.55	0.53	0.31
Position *2*	Cha (1.6)	0.84	0.67	1.34	0.63	0.77	0.53
	Ile (2.5)	1.17	0.97	1.67	0.77	1.24	0.61
	Chg (0.68)	0.58	0.71	0.48	0.58	0.65	0.37
Position *3*	Leu (0.87)	0.47	0.33	0.65	0.54	0.51	0.39
	Val (1.3)	0.48	0.32	0.58	0.41	0.53	0.38
	Met (1.2)	0.47	0.53	0.58	0.50	0.57	0.37

Modifications to compound **1** in both the Northern (positions *1–3*) and Southern (positions *4* and *5*) fragments are indicated. IC_{50} values (μM) for the single change at that position are shown in parentheses. Values with error bars are reported in the Supplemental Data. Nonstandard amino acids are the following: Thx, thyroxine; Bip, biphenylalanine; Chg, cyclohexylglycine; Cha, cyclohexylalanine; $CmeO_2$, methylcysteinesulfone; Paf, 4-aminophenylalanine; Hfe, homophenylalanine; Nal, naphthylalanine.

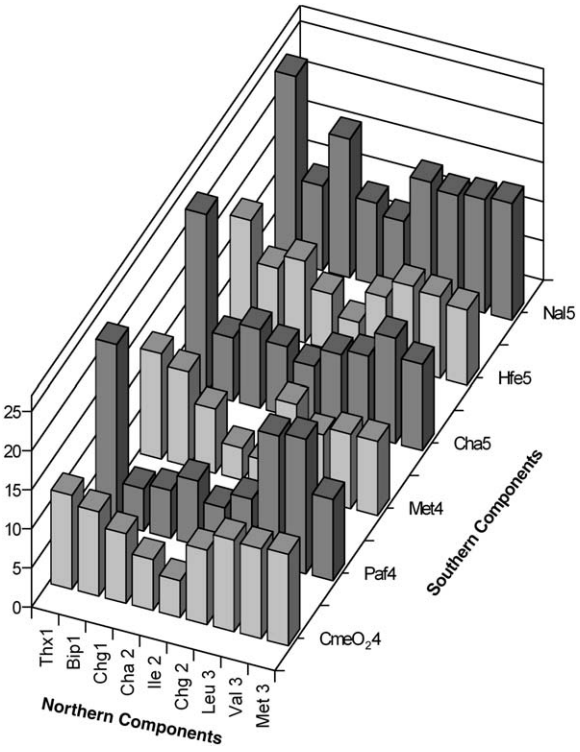


Figure 3. Activity Enhancement of Library Members

The enhancement (x-fold) of activity of each member of the library as compared to compound **1**.

displayed a negative synergistic effect, whereas a significant positive synergistic effect was found with the most improved agent, Chg1Nal5. The observed negative synergistic effect could potentially be due to one mutation causing a change in binding at the site of the second side chain mutation, or it could be due to stabilization

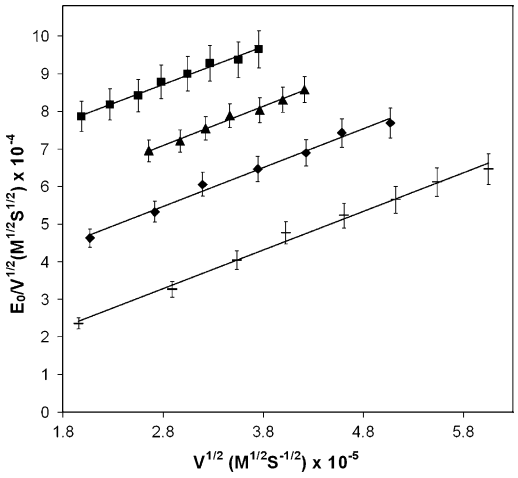


Figure 4. Zhang-Poorman Assay of Compounds Ile2Cha5, Val3-Paf4, and Thx1Nal5

Hyphen, uninhibited HIV-1 PR; diamond, 0.7 μM Ile2Cha5; triangle, 0.3 μM Val3Paf4; square, 0.25 μM Thx1Nal5 (5–40 nM HIV-1 PR, 25 μM substrate).

Table 2. K_i Values of the Doubly Modified Library Members against HIV-1 PR

Northern Component		Southern Component		
Position	Residue	Position	Residue	K_i (nM)
1	Thx	*4*	Paf	29.3 ± 0.2
1	Thx	*5*	Nal	29.2 ± 0.3
1	Thx	*5*	Cha	35.1 ± 0.5
1	Chg	*5*	Nal	65.2 ± 2.3
2	Ile	*5*	Cha	143 ± 1.4
3	Leu	*4*	Paf	40.1 ± 0.7
3	Val	*4*	Paf	51.0 ± 0.5

Modifications to compound **1** in both the Northern (positions *1–3*) and Southern (positions *4* and *5*) fragments are indicated. K_i values are expressed as mean ± standard deviation from triplicate measurements.

of the unbound form of the doubly mutated compound, perhaps by π-π interactions within the biarylether and the aniline side chains of Thx1Paf5.

Significance

We have developed a microtiter plate-based synthetic strategy for our library of doubly modified HIV PR dimerization inhibitors that is high yielding. The excess reagents and byproducts of the reaction were found to have little effect on HIV PR at high concentration. All of the library entries were more potent than starting compound 1, and more than 90% of the compounds were more potent than their respective singly modified parent inhibitors. Some of the more potent agents were further analyzed and were found to be dimerization inhibitors. For this latter group, the binding free energies for the two combined mutations were found to be additive, with two important exceptions. Finally, five of the most potent HIV PR dimerization inhibitors reported to date were identified [13, 14] as a result of this in situ synthesis and screening method.

Table 3. Calculated Increases in Potency of the Doubly Modified Inhibitors if the Two Modifications Had an Additive Effect, as Compared to Inhibitor **1**

Northern Component		Southern Component			
Position	Residue	Position	Residue	Expected Increase[a]	Actual Increase
1	Thx	*4*	Paf	490 ± 50	103 ± 4
1	Thx	*5*	Nal	105 ± 10	102 ± 4
1	Thx	*5*	Cha	117 ± 17	86 ± 4
1	Chg	*5*	Nal	7 ± 1	46 ± 3
2	Ile	*5*	Cha	17 ± 3	21 ± 1
3	Leu	*4*	Paf	95 ± 17	75 ± 3
3	Val	*4*	Paf	40 ± 7	59 ± 2

Modifications to compound **1** in both the Northern (positions *1–3*) and Southern (positions *4* and *5*) fragments are indicated. All values are expressed as mean ± standard deviation from triplicate measurements.

[a] Expected increases were calculated by adding the changes in the free energy of binding (relative to compound **1**) of the two respective individual mutants.

Experimental Procedures

Materials

HIV-1 PR was purchased from Bachem Biosciences (affinity purified grade). Amino acids, coupling reagents, and resins were purchased from either Advanced Chemtech or ChemImpex. All other reagents were purchased from Aldrich and were used without purification.

General Procedure for Synthesis of Northern Peptide Fragments

The Northern tripeptide fragments were synthesized on the Rink amide resin by using a fluorenylmethoxycarbonyl (Fmoc)-based strategy with no side chain protection except for that of Fmoc-Thr(OtBu)-OH. HBTU (O-benzotriazole-N,N,N′,N′-tetramethyluroniumhexafluorophosphate) (3 equivalents) and HOBt (1-hydroxybenzotriazole) (3 equivalents) were used as the coupling reagents with DIEA (3 equivalents) in (DMF) (3.5 ml per 100 mg resin) at room temperature for 3 hr. Upon completion of each coupling step, the terminal Fmoc group was removed with 20% piperidine in DMF (3.5 ml per 100 mg resin), followed by the next coupling reaction. Upon completion of the synthesis, the tripeptides were cleaved from the resin with a trifluoroacetic acid (TFA) cocktail containing 95% TFA, 2.5% triisopropylsilane, and 2.5% water (1.5 ml per 100 mg resin) for 2 hr. The mixtures were filtered through glass wool, and the filtrates were concentrated in vacuo to remove the TFA. The peptides were precipitated with cold ether and stored at −80ºC for 12 hr to insure complete precipitation. The precipitates were collected via centrifugation and were used in the next step without purification.

General Procedure for Synthesis of N-Hydroxysuccinimide-Activated, Acylated Northern Peptide Fragments

The crude Northern tripeptide fragments (**A**, Figure 2) (0.072 mmol) in DMF/CH_2Cl_2 (1:1, 4 ml) were treated with DIEA (4 equivalents) and hexadecanedioic acid bis-(N-hydroxysuccinimide) ester (4 equivalents) at room temperature, and the mixture was allowed to stir for 12 hr. Ethyl acetate (50 ml) was added to each reaction mixture, and the solution was washed with saturated $NaHCO_3$ (2 × 30 ml) and brine. The organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The desired compounds were obtained pure by silica gel chromatography with yields ranging from 51% to 71% (CH_2Cl_2:THF, 10:1–15:1, vv) and were characterized by MALDI mass spectrometry (Supplemental Data).

General Procedure for Synthesis of Southern Peptide Fragments

The Southern dipeptide fragments (**B**, Figure 2) (position *4* mutants) were synthesized in solution by using Fmoc-protected amino acids (1.1 mmol) and H-Phe-OtBu (1.1 mmol) with HBTU (3.3 mmol, 3 equivalents), HOBt (3.3 mmol, 3 equivalents), and DIEA (3.3 mmol, 3 equivalents) in DMF (10 ml) at room temperature for 2 hr. The reaction mixtures were washed with 10% citric acid (2 × 50 ml), saturated $NaHCO_3$ (2 × 50 ml), and brine. The organic layers were dried over anhydrous $MgSO_4$ and concentrated in vacuo. The resulting materials were filtered through a short pad of silica gel (5 cm) with hexane:ethyl acetate (1:1), and the solutions were concentrated in vacuo. The residues were treated with 20% piperidine in DMF (3 ml) for 30 min at room temperature, the solutions were concentrated in vacuo, and the desired products were purified on silica gel (1:1 hexane:ethyl acetate, v/v to 1:10 MeOH:CH_2Cl_2, v/v). The resulting compounds were treated with a solution of TFA and H_2O (95:5, 4 ml) at room temperature for 2 hr. The reaction mixtures were concentrated in vacuo, and the desired materials were purified to homogeneity by reverse-phase HPLC and characterized by ESI mass spectrometry (Supplemental Data).

The Southern dipeptide fragments (position *5* mutants) were synthesized in solution by using Cbz-Asn-OH (1.1 mmol) and t-butyl-protected amino acids (1.1 mmol) with HBTU (3.3 mmol, 3 equivalents), HOBt (3.3 mmol, 3 equivalents), and DIEA (3.3 mmol, 3 equivalents) in DMF (10 ml) at room temperature for 2 hr. The solutions were washed with 10% citric acid (2 × 50 ml), saturated $NaHCO_3$ (2 × 50 ml), and brine. The organic layers were dried over anhydrous $MgSO_4$ and concentrated in vacuo. The resulting materials were filtered through a short pad of silica gel (5 cm) with hexane:ethyl acetate (1:1), and the solutions were concentrated in vacuo. The residues were dissolved in EtOH (15 ml), and Pd/C (10%) was added. The reaction mixtures were stirred under an atmosphere of H_2 at room temperature for 12 hr and filtered through celite, and the solvents were removed under reduced pressure. The resulting compounds were treated with a solution of TFA and H_2O (95:5, 4 ml) at room temperature for 2 hr. The reaction mixtures were concentrated in vacuo, and the desired materials were purified to homogeneity by reverse-phase HPLC and characterized by ESI mass spectrometry (Supplemental Data).

General Procedure for the Microtiter Plate Coupling Reaction

The NHS-activated, acylated Northern peptide fragments (**A**, Figure 2) (20 μl, 50 mM stock solution in DMSO) were added to wells of a microtiter plate that contained the Southern dipeptides (**B**, Figure 2) (20 μl, 200 mM stock solution in DMSO) and DIEA (1.1 μl, 0.0063 mmol) at room temperature. After 12 hr, the reaction mixture in each tube was diluted to a final concentration of 400 μM with DMSO, and the mixtures were analyzed for purity by HPLC and characterized by MALDI mass spectrometry (Supplemental Data). Each stock solution was used directly to obtain inhibitory constants against HIV-1 PR.

Enzymatic Assays

For the determination of IC_{50} values, 50 μl HIV-1 PR (50 nM stock solution in pH 5.5 phosphate buffer A containing 10% glycerol, 0.1% CHAPS, 1 mM EDTA, and 1 mM DTT) was added to the cluster tubes containing a solution (10 μl) of the appropriate amount of the inhibitor stock solution and DMSO. The mixture was incubated at room temperature for 1 hr, and the substrate (40 μl, 150 μM stock in buffer A with 10% DMSO) was added to yield a final substrate concentration of 60 μM. The change in fluorescence at 430 nm (λ_{ex} 360 nm) was monitored at 30ºC for 15 min. For determination of K_i values, all parameters were identical, with the following exceptions: HIV-1 PR concentrations ranged from 5 to 160 nM, and the final substrate concentration was held at 25 μM. All experiments were performed in triplicate.

Supplemental Data

Supplemental Data including a list of compound characterizations and inhibitory activity (IC_{50} values) for purified and in situ-generated inhibitors are available at http://www.chembiol.com/cgi/content/full/13/4/421/DC1/.

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